Review: Assessment of cell proliferation in histological material

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Introduction

There can be little dispute that cellular proliferation is one of the most fundamental of biological processes.¹ Nor can there be disagreement over the importance of assessing cellular proliferation in the study of many biological processes: indeed, Leblond² used such assessment for identifying the three major functional types of cellular population—namely, static, conditional renewal, and continually renewing. The practice of histopathology involves direct or, more usually, indirect assessment of cellular proliferation (and related phenomena such as differentiation) in many situations.³ It is intended that this brief overview will illustrate current ideas about cellular proliferation and its regulation and the advantages and disadvantages of the better known methods for assessing cellular proliferation in histopathological material

(M phase). The S phase is followed by a period of apparent inactivity known as the second gap phase (G_2) which comes before the next mitosis. Interphase comprises successive G_1 , S, and G_2 phases and forms the largest part of the cell cycle. Lajtha proposed that there is a further phase in the cell cycle (G_0) in which cells are not part of the cell cycle but, after suitable stimuli, may rejoin the cycling population.⁶ Not all authors, however, are agreed on the nature of G_0 . In any tissue there are also cells that, for whatever reason, can no longer divide and so any cell population can be divided into a cycling and a non-cycling compartment. This naturally leads to the definition of the proliferative or growth fraction of any cellular population as the ratio of cycling to cycling plus non-cycling cells.⁷

Although there is a great deal which remains unclear, large advances have been made in our understanding of the regulation of the eukaryotic cell cycle. At a simple phenomenological level molecules can be identified, especially proteins, whose functional state varies in synchrony with the cell cycle, either in protein level or in activity.^{8,9} In model systems such as yeast, cell cycle mutants can be identified by conventional and molecular genetic analyses, and these approaches have led to the cloning and sequencing of genes involved in the cell cycle. Biochemical methods have also been used to define enzymatic activities involved in various phases of the cell cycle, such as in DNA

The cell cycle

The observations of Howard and Pelc, who used ^{32}P incorporation and autoradiography during the early 1950s, led to the introduction of the concept of the cell cycle and its subdivision into several phases (figure).^{4,5} DNA synthesis and doubling of the genome take place during the synthetic or S phase. This is preceded by a period of variable duration known as the first gap (G_1) phase which separates the S phase from the previous mitosis

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Figure The cell cycle, adapted from Wright.¹ It should be remembered that there may be considerable variation in the duration of phases of the cell cycle within and between tissues, both normal and neoplastic.^{1,15,16}

synthesis. The results of such strategies indicate that the molecular control of cellular proliferation has been remarkably conserved through evolution with similar regulatory mechanisms operating in all eukaryotes, with cascades of regulatory genes which seem to have a role in controlling cell division.⁹⁻¹⁰ For example, a key gene in yeast and in man encodes a 34 kilodalton protein kinase, essential for cell division, known as *cdc2*.¹¹⁻¹³ The level of this protein does not change during the cell cycle but its functional activity does as a consequence of altered phosphorylation¹⁴⁻¹⁵; phosphorylation of target proteins, which changes their function, is a particularly prevalent regulatory mechanism in cell cycle control.

Other important cellular proteins involved directly or indirectly in the control of cell proliferation have been identified. For example, cyclins are a group of proteins (also known as proliferating cell nuclear antigens or PCNA), which accumulate progressively through interphase and disappear at the end of mitosis. These proteins are now known to be components of DNA polymerase δ and are essential for completion of the cell cycle.⁹⁻¹⁶⁻¹⁷ These occur throughout eukaryotes, including plants.¹⁸ Several histone proteins vary in synchrony with the cell cycle and could be suitable markers for assessing changes associated with cell proliferation.¹⁹ The regulation of genes related to the cell cycle by specific promoter sequences is also now becoming better understood.²⁰ Finally, there seem to be specific genes associated with growth arrest in mammalian cells, although these are as yet only poorly characterised.²¹⁻²²

There seem to be numerous functionally interrelated substances that vary in level or activity during the cell cycle, whose functions (whether structural or regulatory) are to permit the initiation and completion of normal cell division. Against this background a variety of cellular changes can be identified which can be used to pinpoint cell proliferation in histological material. In simple terms, mitoses can be counted, the incorporation of nucleotides (or their analogues) into newly synthesised DNA during the S phase can be identified, and the varying levels of structural or functional moieties associated with different aspects of the cell cycle can be assayed. Antibodies have been raised to the products of several *cell division cycle* (*cdc*) genes in yeast and can detect the relevant protein in both biochemical assays and immunohistochemical preparations.¹⁰⁻²³ Antibodies have been used to show the presence of cyclin/PCNA and other gene products related to the cell cycle such as DNA polymerase α . Many other cell cycle related proteins will also probably be useful in the histological assessment of cell proliferation or exit from the proliferative compartment.

Methods for assessing cellular proliferation

MITOSES

The mitotic phase of the cell cycle is the only

part that can be recognised by simple morphological examination, and the reader is referred to standard texts for the classic morphological features of the mitotic cycle.¹ As these features are so well described the assessment of cell proliferation by counting mitoses should be straightforward; this method is considered to provide useful information in many tumours such as lymphomas,²⁴⁻²⁵ melanomas,²⁶ soft tissue sarcomas,²⁷ especially smooth muscle tumours.²⁸ It is part of the grading system of several tumours, notably carcinoma of the breast.

Several potential problems must be considered in the use of mitoses as an indicator of proliferation. The identification of mitoses can be very difficult; careful microscopical examination is essential, only those cells that are unequivocally in mitosis are acceptable for counting. Pyknotic nuclei and suspicious cells should be excluded.²⁸⁻³⁰ When the number of mitoses per high power field is counted, the area of the high power field should be given;³¹⁻³² preferably the number of mitoses per 1000 cells should be stated. The process of mitosis in inadequately fixed material may go on to completion, while few if any new mitoses will be initiated, leading to a considerable underestimate of the true numbers of mitoses and apparent regional variations.³²⁻³³

DNA CONTENT

Another approach to assessing cell proliferation is to take advantage of the process of DNA synthesis. A normal somatic cell has a diploid genome; DNA synthesis during S phase leads to a doubling of the genetic information and the cell becomes temporarily tetraploid. This process can be measured in several ways.

Thymidine labelling

The introduction of tritiated thymidine and the development of high resolution autoradiography during the 1950s led to the use of thymidine labelling as the "gold standard" for cell kinetic studies. The method requires viable cells to take up tritiated thymidine and for this to be incorporated into DNA during the S phase. It consequently requires the *in vivo* administration of radiolabelled thymidine which can rarely be justified in clinical practice. Alternatively, biopsy and other specimens must be incubated with tritiated thymidine for some time *in vitro* before fixation. Both of these restraints militate against thymidine labelling as a practical routine procedure for histopathologists. In some centres, however, thymidine labelling has been extensively used on clinical material from a wide range of conditions,³⁴⁻³⁶ particularly lymphomas³⁷⁻³⁹ and breast carcinoma.⁴⁰ Several authors have reported that thymidine labelling provides information of prognostic relevance in non-Hodgkin's lymphomas⁴¹⁻⁴³ and the demonstration of incorporation of tritiated thymidine by scintillation counting seems to provide similar information.⁴⁴⁻⁴⁵ Studies of breast carcinoma indicate that thymidine labelling indices closely correlate with several pathological and clinical variables and that it is a predictor of

biological behaviour.^{40 46 47} In other tumours the clinical importance of thymidine labelling indices has been less clear cut.⁴⁸

Bromodeoxyuridine incorporation

Synthesis of DNA can also be assessed by measuring the incorporation of a thymidine analogue such as bromodeoxyuridine. This molecule can then be detected by specific antibodies,^{49 50} either in tissue sections or by flow cytometry,^{50 51} and used for detailed cell cycle analysis⁵² as with thymidine incorporation. The disadvantages are similar to those of tritiated thymidine, in particular the need for in vivo administration or in vitro incubation. Ethical permission for some in vivo studies has been obtained, however, and in vitro incubation of biopsy or other surgical specimens is feasible.⁵³⁻⁵⁵

Flow cytometry

The capacity of some dyes to bind to DNA in a stoichiometric manner means that the amount of DNA present in a nucleus can be determined, at least in comparison with some reference standard. The method of static cytometry has been widely used to investigate the relative DNA content of cells, but only relatively limited information can be obtained and that relates to ploidy rather than proliferation itself. Considerably more information can be obtained using flow cytometry in which large numbers of cells are analysed and appropriate statistical methods applied to the resulting data to give information about DNA content and cell cycle variables.

The principles of flow cytometry have been reviewed elsewhere.^{56 57} In brief the procedure involves the examination of a single cell suspension passing a given point, by a suitably tuned laser beam, with reflected or transmitted light, or both, being detected by suitably placed detectors and converted into an electronic signal. Many variables can be assessed—for example, the presence on the cell of a fluorescent dye linked to an antibody, or the fluorescence due to a dye bound to nucleic acid. Information about the number of cells and their DNA content can be obtained from these data, providing kinetic information if suitably analysed.⁵⁸ In the absence of aneuploidy cells in G_0 , G_1 will be diploid, cells in G_2 will be tetraploid, and cells in S phase will have intermediate amounts of DNA. Assessment of kinetic variables is considerably complicated by the presence of aneuploidy,⁵⁹ but with suitable mathematical analysis, the S phase fraction can be determined in most such cases.^{60 61}

Of particular interest is the fact that DNA content can be measured in nuclei recovered from fixed and paraffin wax embedded histopathological material.^{62 63} Many different pathological conditions have been studied with this technique, but as with other methods of assessing cell proliferation, much attention has been given to lymphoid neoplasms including Hodgkin's disease⁶⁴ and non-Hodgkin's lymphomas⁶⁵⁻⁶⁸. Flow cytometry has also been applied to cytological preparations.⁶⁹ Some

studies have shown that flow cytometric assessment of proliferative indices has prognostic value⁷⁰⁻⁷⁴ and can predict the response to treatment, particularly in high grade tumours⁷⁵⁻⁷⁷; it may also be useful within histological categories such as centroblastic-centrocytic lymphoma.⁷⁸ Flow cytometric studies of breast carcinoma have been reported⁷⁹⁻⁸² and some have indicated that this method of assessing cell proliferation provides prognostic information,⁸³⁻⁸⁵ although this is, predictably, correlated closely with histological tumour grade. Studies of other tumours such as colorectal cancer,⁸⁶ gastric cancer and precancer,⁸⁷ lung tumours,⁸⁸ and melanoma⁸⁹ have been reported.

The most important disadvantages of flow cytometry include the cost of the equipment required, the need to disrupt tissues so that the spatial relation of cellular subpopulations is lost, and the possible inclusion of admixed non-neoplastic cells in samples. On the other hand, the information obtained is objective and represents measurements on very large numbers of cells, in contrast to most of the other methods described for the investigation of cell proliferation.

IMMUNOHISTOCHEMICAL METHODS

As well as their use in the immunohistochemical demonstration of bromodeoxyuridine, antibodies have become an important means of assessing cell proliferation. A particular advantage is that the immunohistological demonstration of cell cycle related antigens allows spatial orientation to be shown, and the phenotype of proliferating cells can be determined by double staining methods. C_5F_{10} , an antibody, which recognises cells in mitosis has been described⁹⁰ but this has been found to cross-react with many other cellular components and offers few advantages over conventional assessment of mitoses in histological sections (P A Hall, unpublished observations).

Perhaps the best known antibody that recognises proliferating cells is Ki67. This identifies a poorly characterised nuclear antigen associated with the cell cycle, being expressed in all phases except G_0 .^{91 92} Recent studies using a variety of techniques, including confocal microscopy, suggest that the antigen is a component of the nuclear matrix.^{93 94} Several studies have shown that there is a close correlation between Ki67 immunoreactivity and other variables of cellular proliferation such as thymidine labelling,⁹⁵⁻⁹⁸ bromodeoxyuridine incorporation,^{96 99-102} and flow cytometry.^{95 103 104} There is evidence, however, that in nutritionally deprived cells (which are not uncommon in tumours) there can be a discrepancy between Ki67 immunoreactivity and other variables of cellular proliferation.^{94 105 106} Nevertheless, Ki67 immunostaining has been extensively used as an operational marker of cellular proliferation and, although certain caveats have been raised, will probably remain a popular method. A major limitation to its use, however, has been the need for fresh or snap frozen material as the antigen is very sensitive to fixatives and may deteriorate with time even when stored at -20° .¹⁰⁷

Ki67 immunostaining has been used in numerous disease states including tumours such as non-Hodgkin's lymphomas,^{100 108 109} Hodgkin's disease,^{110 111} plasma cell tumours,¹¹² breast cancer,¹¹³⁻¹¹⁵ soft tissue tumours,¹¹⁶ brain tumours,^{117 118} lung,¹¹⁹ cervical¹²⁰ and colorectal¹²¹ carcinomas and liver tumours.¹²² Ki67 immunoreactivity seems to provide prognostically useful information in lymphoma,^{100 109 123 124} and this may also be the case in breast cancer.¹²⁵ As with other clinicopathological studies of cell proliferation, differences in quantitation, presentation of the results, and most importantly, the way in which the patients were treated make direct comparisons of these studies difficult. Ki67 immunostaining has also been applied to non-neoplastic conditions including reactive lymphoid lesions,^{126 127} renal biopsy specimens from transplant patients and cases of glomerulonephritis,¹²⁸ inflammatory bowel disease,¹²⁹ biopsy specimens of synovia in degenerative joint disease,¹³⁰ endometrial stroma,¹³¹ and sarcoïdosis,¹³² and in both histological and cytological material.^{111 133 134}

Another approach has been to use antibodies that recognise some component of the cellular DNA replication machinery such as DNA polymerase α , which is transcriptionally regulated in a cell cycle dependent manner.¹³⁵ This enzyme can be recognised in cytological and histological material by monoclonal antibodies,^{136 137} and these have been successfully applied to a variety of clinical specimens, including lymphomas.^{138 139} Other authors have proposed that the immunohistological demonstration of transferrin receptor (CD71) can be used as an index of cellular proliferation and can provide useful clinical information, at least in non-Hodgkin's lymphoma.¹⁴⁰ The transferrin receptor is expressed widely, not only on proliferating tissues but also on those that are non-proliferating but metabolically active,¹⁴¹ and is thus a poor index of cellular proliferation.¹⁴²

Autoantibodies which react with nuclear antigens in proliferating cells are found in some patients with connective tissue diseases, and the possibility that they may be useful biological markers for the study of proliferating cells has been recognised for some time.^{143 144} One particular group of autoantibodies recognises cyclin/PCNA.¹⁴⁵ Human antibodies, or murine monoclonal antibodies which recognise cyclin/PCNA, have been used in cytological¹⁴⁶ and histological material and have recently been reported to provide a simple method of recognising proliferating cells in methacarn or alcohol fixed, wax embedded tissue, with results comparable with those achieved with flow cytometry.¹⁴⁷ Other nuclear antigens have been reported whose expression is temporarily related to cellular proliferation, and some are localised in the nucleolus. It seems very likely that antibodies which recognise other structural or functional molecules regulated during the cell cycle will become available.

NUCLEOLAR ORGANISER REGIONS

Nucleolar organiser regions represent loops of

DNA which have the genes for ribosomal RNA, together with associated proteins,¹⁴⁸ although the function of these nucleic acid-protein complexes is not fully understood. The proteins shown by the argyrophilic method are possibly related to RNA polymerase I¹⁴⁹ or other proteins such as C₂₃ (nucleolin)¹⁵⁰⁻¹⁵² and B₂₃ protein.¹⁵² The exact function of these proteins remains uncertain but they may maintain DNA in an extended configuration¹⁵³ or have some regulatory function in controlling the transcription of the genes for ribosomal RNA.¹⁵⁴⁻¹⁵⁶

The assessment of silver stained nucleolar organiser regions (AgNORs) in histopathological material has been popularised by Crocker,^{148 157} who has applied methods that had been used extensively in cytogenetics for many years. Simple silver staining methods can be used to show the presence of proteins associated with nucleolar organiser regions in interphase chromosomes in conventional histological sections, and these can be counted.¹⁵⁸ As nucleolar organiser regions are associated with acrocentric chromosomes it was initially thought that these data would largely reflect ploidy, but correlation with conventional histological variables,¹⁴⁸ with Ki67 immunoreactivity,¹⁵⁹ and with flow cytometry¹⁶⁰ suggested that AgNORs are more closely related to cell proliferation than to ploidy. Recent studies of interphase nuclei with morphological,¹⁶¹ cytogenetic,¹⁶² and cell kinetic¹⁶³ methods suggest that the number of interphase nucleolar organiser regions is strictly correlated with cell proliferation.

Recently there have been many studies describing the application of silver staining methods for the demonstration of AgNORs in histological material from a wide range of diseases.¹⁴⁸ Assessment of AgNORs seems to give similar information as other indices of cellular proliferation in lymphomas (Hall PA, Crocker J, Murray BG, Levison DA, unpublished observations).^{157 159} Nucleolar organiser region staining is claimed to be of diagnostic value in numerous conditions including childhood tumours,¹⁶⁴ soft tissue tumours,¹⁴⁸ melanocytic and other cutaneous lesions,¹⁶⁵ and tumours of breast,¹⁶⁶ lung,¹⁶⁷ salivary gland,¹⁶⁸ cervix,¹⁶⁹ central nervous system, liver, and gastrointestinal tract.^{148 170} Nucleolar organiser regions can also be shown in cytological preparations and AgNOR staining can be combined with immunohistochemistry.^{164 171}

In some tissues such as thyroid¹⁷² nucleolar organiser regions are reported to be less useful and some studies suggest that the counting of AgNORs may not provide pathologists with additional information.^{173 174} With the exception of preliminary reports on neuroblastoma and other childhood tumours,¹⁷⁵ little information on the prognostic importance of AgNOR staining is available. The present evidence suggests that nucleolar organiser regions represent a means of assessing cellular proliferation, but a better understanding of nucleolar organiser regions and their component proteins is vital for the rational interpretation of this method. Further studies are needed

to substantiate early claims that assessment of nucleolar organiser regions may be useful in histopathological diagnosis.

Quantitation

Irrespective of the method used for the assessment of cell proliferation, it is important to consider how it should be quantitated. Flow cytometry analyses very large numbers of cells, but considerably fewer are generally examined in the other methods. For the immunohistochemical assessment of proliferation, some authors have used manual counting of cells on tissue sections, and this may be very time consuming as many cells must be counted.^{109 113 121} There is no absolute answer to the question, how many cells need to be counted? In fact, the more pertinent question is how many cells need to be counted to be able to distinguish confidently between two values? This topic is well reviewed elsewhere.^{176–178} An alternative is to use a semiquantitative method and this seems to give reproducible results.^{119 121 178} Image analysis techniques have also been successfully applied to sections and fine needle aspirates immunostained with Ki67.^{115 179 180}

Heterogeneity within a specimen must also be considered, particularly in tumours.^{35 113 121 181 182} there are functionally and kinetically distinct populations in normal and pathological tissues.^{36 182 183} For example, there is good evidence that stem cells are present in tumours and these may represent the predominant biologically and therapeutically relevant population.^{35 184–186} Consequently the assessment of cell proliferation in different subpopulations may provide more meaningful information than crude assays that use all the cells in the sample as the denominator when deriving proliferation indices. In situ methods such as immunohistochemical techniques combined with double staining methods for phenotypic markers may provide new approaches to the functional analysis of the proliferative characteristics of tissues, particularly neoplasms. Heterogeneity may extend to there being different kinetic indices in tumour samples from different sites,¹⁸⁷ and this and other aspects of kinetic heterogeneity may have considerable therapeutic implications.¹⁸⁸

“Rates not states”

The methods for assessing cellular proliferation so far described provide information about the proportion of cells cycling at any given moment—that is, the *state of proliferation*. Such measurements provide no information about the *rate of proliferation* which requires measurement of (i) the rate at which cells enter the cycle and (ii) how long they remain there. The importance of the distinction between rates and states of proliferation is illustrated by a simple example. If the length of time spent in the cell cycle is long, then many cells will appear to be in cycle at any given moment, although the population may be proliferating quite slowly. Consequently information about proliferative rates can be more informative

than proliferative states. Such information is, in general, more difficult to obtain.

Several methods can be used to obtain information about proliferative rates.^{35 36 177} These include metaphase arrest or stathmokinetic methods to determine the rate of mitosis, or labelling methods using pulses of thymidine and multiple biopsy specimens, double labelling or continuous labelling methods, and the fraction of labelled mitosis technique. These complex and time consuming methods are rarely practical in clinical material due to the need for the in vivo administration of toxic substances or the need for in vitro incubations. They also require multiple biopsy specimens in most cases. Thornton, however, has recently reported the application of a double labelling method, with both thymidine and bromodeoxyuridine incorporation, for the measurement of the S phase duration in endometrial biopsy specimens,¹⁸⁹ and other studies of clinical material have been reported. Differences within and between tumours in the duration of the cell cycle may also be of clinical importance, yet are not shown by simple “state” measurements.³⁵ Cell loss is also an important component of the kinetic state of tissue, including tumours, but this is difficult to quantify.^{35 190} The assessment of numbers of apoptotic bodies provides an index of the state of cell death, but information on rate of cell death is more difficult to obtain.¹⁹¹ Loss of cells from the proliferative compartment—for example, by differentiation—is also difficult to assess.

Conclusions and prospects

The assessment of cellular proliferation in histological material is a valuable component of conventional histopathological analysis, and a variety of methods can be used to quantify this. Ideally such methods should be simple, reproducible, and applicable to conventionally processed histological and cytological preparations. The method should be relatively inexpensive and interpretation of the results should be straightforward. Many of the different methods described in this review measure slightly different variables, and although comparable, the data produced are not identical. It is always useful to consider the nature of the biological process being used as an index of proliferation. With mitoses, the S phase fraction, and other markers of DNA synthesis this is quite simple, but with AgNORs and Ki67 immunostaining, we remain uncertain as to exactly what is being measured. At present the available methods all have some disadvantages.

It should be remembered that cell proliferation is a normal biological process. Many normal tissues are actively proliferating—for example, germinal centres and colonic and small intestinal crypt epithelium. As a consequence the interpretation of indices of cell proliferation should always be in the context of knowledge of the proliferative nature of the normal tissue. Such an argument leads to the inevitable conclusion that methods which permit in situ assessment of cellular proliferation, such as mitosis counting or immunohisto-

chemical methods, may be preferable to those methods that require the disruption of tissues such as flow cytometry. The identification and cloning of genes and their products which regulate the cell cycle may lead to the analysis of cell proliferation in histological material by the use of peptide specific antibodies directed at these molecules.

Assessment of cell proliferation has numerous applications. Many are already well defined, as in the counting of mitoses in smooth muscle tumours. There is considerable evidence that assessing cellular proliferation in a variety of tumours provides useful information and may be of major prognostic importance. Further studies along these lines with careful clinicopathological correlation are needed to confirm such ideas, but multivariate analyses are urgently required to establish that cell proliferation provides information independent of other clinical and histological variables. For example, O'Rielly *et al* have shown that S-phase fraction determined by flow cytometry is of prognostic importance in breast cancer, but that in a multivariate analysis histological grade and S-phase fraction seemingly provide equivalent information.⁸⁵ Moreover, despite the numerous studies reporting the assessment of cell proliferation in tumours, it does not yet directly influence patient management, with the possible exception of smooth muscle tumours. The analysis of cell cycle times and correlation with clinical variables are needed, and the possibility that assessment of cellular proliferation during and after treatment may give clinically useful information about response should be considered. Given the extensive phenotypic and kinetic heterogeneity seen in tumours, studies to define proliferative capacity in phenotypically defined subpopulations, such as those containing stem cells, may provide information of more biological and clinical relevance than simple measures of proliferation. Finally, the application of these methods to non-neoplastic conditions—for example, in the study of glomerulonephritis and transplant rejection in renal biopsy specimens—are areas that should be explored.

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